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Kean, R., McKloud, E., Townsend, E. M., Sherry, L., Delaney, C., Jones, B. L., Williams, C. and Ramage, G. (2018) The comparative efficacy of antiseptics against *Candida auris* biofilms. *International Journal of Antimicrobial Agents*, 52(5), pp. 673-677.

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Deposited on: 28 August 2018

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Accepted Manuscript

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PII: S0924-8579(18)30138-9
DOI: [10.1016/j.ijantimicag.2018.05.007](https://doi.org/10.1016/j.ijantimicag.2018.05.007)
Reference: ANTAGE 5446



To appear in: *International Journal of Antimicrobial Agents*

Received date: 20 December 2017
Revised date: 26 March 2018
Accepted date: 8 May 2018

Please cite this article as: Ryan Kean , Emily McCloud , Eleanor M Townsend , Leighann Sherry , Christopher Delaney , Brian L Jones , Craig Williams , Gordon Ramage , The comparative efficacy of antiseptics against *Candida auris* biofilms, *International Journal of Antimicrobial Agents* (2018), doi: [10.1016/j.ijantimicag.2018.05.007](https://doi.org/10.1016/j.ijantimicag.2018.05.007)

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Highlights

- *C auris* biofilms display decreased sensitivity to skin disinfection agents
- *Candida* species were differentially sensitive to antiseptics
- Hydrogen peroxide treatment was ineffective against *Candida auris* biofilms

ACCEPTED MANUSCRIPT

The comparative efficacy of antiseptics against *Candida auris* biofilms

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RUNNING TITLE: Antiseptics against *Candida auris* biofilms

Abstract

Candida auris has emerged as a significant clinical entity due to its ability to cause outbreaks within the healthcare setting. A key feature of its nosocomial lifestyle is its ability to transfer between patients, yet little is known about the mechanisms behind this. A panel of *C. auris* clinical isolates was screened for their planktonic and sessile susceptibilities to skin disinfection challenge using povidone iodine, chlorhexidine and hydrogen peroxide. *C. auris* biofilms displayed increased tolerance to these strategies compared to planktonic cells. Additionally, analysis using a complex biofilm model demonstrated reduced susceptibility against clinically relevant concentrations of chlorhexidine and hydrogen peroxide, with complete eradication achieved only using povidone iodine. Principal component analysis (PCA) also revealed distinct clustering of *C. auris* biofilms in comparison to *C. albicans* and *C. glabrata* biofilms, and directionality with respect to different treatments. These findings indicate differential responses of different *Candida species* with respect to antiseptic challenge against biofilms, with *C. auris* appearing to be more resilient as a complex community.

KEYWORDS: *Candida auris*, biofilm, resistance, antiseptics, decolonisation

1. Introduction

Since its first report in 2009, the emerging multidrug-resistant pathogenic yeast *Candida auris* has attracted considerable attention as a source of healthcare associated infections. Alarming, it has been attributable for a number of nosocomial outbreaks globally, including a UK intensive care unit, whereby almost 20% of colonised patients developed candidaemia [1]. High transmission within critical care units has been reported, with environmental sampling yielding *C. auris* from beds and chairs, in addition to multiuse patient equipment [2, 3]. The mechanism behind its persistence within the healthcare environment is unknown, yet it is thought to be multi-faceted, whereby it subsequently contaminates the patient and healthcare environment. The primary site of colonisation in patients remains unknown, though it has been isolated from various sites of the skin and has also been isolated 3 months after initial identification despite antifungal treatment and negative screens [3]. Within the environment, it has been shown to survive and persist for prolonged periods on a variety of fomites [4, 5]. Disinfection regimens are also problematic, with various studies identifying variable and unsatisfactory outcomes [6-8]. Potentially linked to its survival profile is the organisms' ability to exist as a biofilm. Despite biofilm formation initially being dismissed [9], more recent studies have demonstrated that *C. auris* can form highly resistant biofilms [10, 11]. Several studies have assessed the fungicidal efficacy of antiseptics against planktonic *C. auris* cells [7, 12], however there are minimal data evaluating their effectiveness against biofilms. This study used a three-dimensional complex biofilm model to investigate the efficacy of a panel of antiseptic therapeutics including povidone iodine (PVP-I), chlorhexidine (CHX) and hydrogen peroxide (H₂O₂). The data presented herein, supports the hypothesis that the ability of *C. auris* to form biofilms may be a potential mechanism that results in reduced susceptibility to antiseptic agents.

2. Materials and methods

2.1 Strains and culture conditions

Four *C. auris* isolates (NCPF 8971, NCPF 8973 [non-aggregative], NCPF 8977, NCPF 8978 [aggregative]) and *C. albicans* ATCC 10231 and *C. glabrata* ATCC 2001 were used throughout this study, as previously described [11, 13]. Isolates were stored and maintained on Sabouraud dextrose (SAB) agar (Oxoid, Hampshire, UK) prior to propagation in yeast peptone dextrose (YPD) (Sigma-Aldrich, Dorset, UK) medium for 16-18 h, at 30°C with gentle agitation. Cells were then washed by centrifugation in phosphate buffered saline ([PBS] Sigma-Aldrich, Dorset, UK), before being standardised to assay specific concentrations after counting using the haemocytometer.

2.2 Two-dimensional biofilm formation and susceptibility testing

For biofilm development, organisms were standardised to 1×10^6 cells/mL in RPMI-1640 (Sigma-Aldrich, Dorset, UK) and grown at 37°C for 24 and 48 h in 96-well flat-bottomed microtitre plates (Corning, NY, USA). Following growth, biofilms were washed with PBS to remove non-adherent cells and treated with serially two-fold diluted concentrations of CHX (1 - 0.002%), PVP-I (10 - 0.02%) and H₂O₂ (1 - 0.002%). Following exposure for 5, 10 and 30 min, the antiseptics were neutralised for 15 min using 5% sodium thiosulphate (Fisher Scientific, Loughborough, UK). For both planktonic and sessile cells, minimum inhibitory concentrations (MIC), were calculated using the XTT [2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2Htetrazolium-5-carboxanilide] assay, with the MIC recorded as the concentration which reduced metabolic activity by 90%, as previously described [14].

2.3 Preparation of hydrogel and biofilm development on cellulose matrix

Next, the impact of the therapeutics against *C. auris* NCPF 8971, *C. auris* NCPF 8978, *C. albicans* and *C. glabrata* were assessed using a complex wound biofilm model

developed in house [15]. Briefly, hydrogels were prepared using 10% 3-sulfopropyl acrylate potassium salt, 0.95% poly(ethylene glycol) deacrylate, 0.01% 1-hydroxycyclohexyl phenyl ketone, with the addition of 50% horse serum (Thermo Fisher Scientific, Loughborough, UK) in sterile water to the final volume. Gels were then polymerised in 12 well plates (Corning, NY, USA) using a 366nm ultraviolet lamp for 30 min. For mature biofilm formation, the isolate was standardised to 1×10^7 cells/mL in PBS and added to sections of cellulose matrix (1.25 cm^2) prior to incubation for 90 min at 37°C , with gentle agitation. Sections were then placed on top of the hydrogel and incubated for a further 48 h. In addition, early biofilms were grown for 90 min by incubating standardised cells on the cellulose matrix, as described above.

2.4 Complex biofilm susceptibility testing

Following growth in the complex model, biofilms were washed with PBS to remove non-adherent cells. Biofilms were then challenged with clinically relevant concentrations of CHX (0.05%, 2%), PVP-I (10%) and H_2O_2 (3%) for 5 min, before being neutralised, as described above. Following neutralisation, matrices were removed, sonicated at 35 kHz for 10 min in an ultrasonic water-bath (Fisher Scientific, Loughborough, UK) to remove biofilm cells, with cellular viability determined using the Miles and Misra plate counting method [15].

2.5 Scanning Electron Microscopy (SEM)

Early (90 min) and mature (48 h) biofilms of *C. auris* NCPF 8971 were grown on cellulose matrix as stated above and visualised for SEM as described previously [15]. Briefly, biofilms were washed with PBS before fixation using 2% glutaraldehyde, 2% paraformaldehyde and 0.15% alcian blue in 0.15M sodium cacodylate and processed for SEM. The samples were sputter coated with gold before being visualised using a JEOL JSM-6400 scanning electron microscope.

2.6 Statistics

Data distribution, statistical analysis and graph production were performed using GraphPad Prism (version 8; La Jolla, CA, USA). Statistics were calculated based from \log_{10} reductions from the untreated controls. Data were assessed for normality before student t-tests were used to compare treated and untreated samples, with statistical significance was achieved if $p < 0.05$. Principle component analysis (PCA) was performed on \log_{10} reduction values with R software using in-built functions. Clustering was performed using the partitioning around medoids (pam) algorithm using the R package 'cluster'. Visualisation by the package 'ggplot2' was utilised to provide figures.

3. Results

Initially, the antiseptic efficacy of three agents were tested against four *C. auris* isolates using a 96 well microtitre plate methodology. When biofilms were treated with PVP-I for 5 min, concentrations of 1.25-2.5% were required to inhibit biofilms, a 16 to 128 fold change compared to planktonic cells (Fig 1A). Increasing the exposure time (10 and 30 min) was shown to increase susceptibility to 0.625-1.25%, 8 to 64 fold change compared to planktonic cells. CHX was highly active against planktonic cells, whereas biofilms were less susceptible with MIC's increasing 2 to 16 fold (Fig 1B). Elevated biofilm MIC's were also observed following H₂O₂ exposure, with concentrations ranging between 0.25 - >1% required to kill biofilms, i.e. 16-fold increase in the planktonically active concentration (Fig 1C). Regardless of the antiseptic active used, minimal differences in susceptibility were observed between 24 and 48 h biofilms. Likewise, minimal variation was observed between all four *C. auris* isolates. Strains NCPF 8971 (non-aggregative) and NCPF 8978 (aggregative), two phenotypic and genotypically distinct isolates, and comparator species *C. albicans* and *C. glabrata* were then selected for further experimentation using a complex biofilm model.

Early (90 min) and mature (48 h) biofilms were grown before treatment with clinically relevant concentrations of each of the antiseptics. When early biofilms were treated with 0.05% CHX, mature biofilms demonstrated decreased susceptibility across all tested isolates ($p < 0.001$). Both *C. auris* isolates were less susceptible ($< 1 \log_{10}$ reduction), compared to 2- and 3- \log_{10} reduction for *C. albicans* and *C. glabrata* respectively. When the CHX concentration was increased to 2%, complete eradication was shown against all early biofilms. This same trend was observed against mature biofilms of *C. albicans* and *C. glabrata*, however $\sim 10^3$ and $\sim 10^2$ CFU/mL remained viable post treatment for *C. auris* NCPF 8971 (Figure 2A) and NCPF 8978 (Figure 2B) respectively. Visually, CHX treated *C. auris* NCPF 8971 biofilms appeared to fuse and form a coating on the cells (Fig 3C, D), with only the top layer of the mature biofilm shown to be covered with CHX (Fig 3H, I). The least effective treatment against *C. auris* was 3% H₂O₂, with only a ~ 1 - \log_{10} reduction observed against both *C. auris* isolates early and mature biofilms (Figure 2A, B). This treatment was more effective against *C. albicans*, achieving complete

eradication against the early biofilms, however $\sim 10^2$ CFU/mL remained viable for treated mature biofilms ($p < 0.001$, Figure 2C). Interestingly, early biofilms of *C. glabrata* were significantly more resilient than mature biofilms against this treatment ($p < 0.001$, Figure 2D). The most effective treatment strategy was 10% PVP-I, to which regardless of *Candida* isolate or biofilm phase, was able to achieve complete eradication (Figure 2 A,B,C,D). When analysed visually, residual active agent can be observed encasing the treated biofilm cells of *C. auris* NCPF 8971 (Fig 3B, G).

The PCA plot (Fig 2E), illustrates clustering of mature biofilms of each of the tested isolates and each treatment parameter. Clustering analysis demonstrates two distinct clusters between both *C. auris* isolates (cluster 1) and between *C. albicans* and *C. glabrata* (cluster 2). Factor loading plots of each treatment demonstrate the contribution of the variance on each of the components. The variance between the two clusters is seen here to be due to difference to susceptibility between treatments parameters. *C. albicans* and *C. glabrata* cluster according to susceptibility to H_2O_2 and CHX 0.05%, both of which were ineffective against *C. auris*.

4. Discussion

Given the multidrug resistant phenotype of *C. auris* its control within the environment is paramount. Its environmental persistence is a key attribute within its pathogenic repertoire, and is responsible for a number of outbreaks reported globally [16]. Despite being primarily associated with a number of life-threatening, invasive infections including wound and bloodstream, it appears also to readily colonise the skin. Additionally, person-to-person transmission between close contact individuals of the patient is possible, with as little as 4 hours required for the organism to successfully colonise [1]. Currently, no known guidelines exist for the successful decolonisation of *C. auris*. Several *in vitro* investigations have established the susceptibility of the organism to CHX [11, 12]; this however has not translated clinically, and patients who are receiving daily CHX bathing continued to have *C. auris* isolated from their skin [1]. Indeed, Moore *et al* (2017) demonstrated formulation dependent sensitivity, with >5 log reductions only observed in 2% CHX with the presence 70% isopropyl alcohol [7]. This study however investigated suspension testing, therefore the effectiveness of this combination against adherent *C. auris* cells remains unknown. Furthermore, patients can remain colonised even following antifungal exposure, with *C. auris* isolated from skin and urine post-prolonged echinocandin therapy [17]. Given the propensity of *C. auris* to form biofilms [11], and its isolation from catheter tips [1, 18], skin decolonisation prior to invasive therapy could be considered.

Interestingly, PVP-I, a commonly used pre-surgical wash, was shown to be equally active against both early and mature biofilms when assessed by culture. This is in agreement with other studies, whereby PVP-I demonstrated excellent fungicidal activity against *C. auris* [7, 12]. The use of 10% PVP-I for surgical skin preparation has been used clinically for *C. auris*, with no reported postoperative infection developed [12]. An interesting finding from this study was the ineffectiveness of H₂O₂ against both planktonic and sessile cells of *C. auris*. These findings differ from those of previous studies, whereby H₂O₂ demonstrated significant fungicidal activity [8, 12]. Discrepancies between these findings are likely due to the test methodologies employed, with vaporised H₂O₂ assessed in one of these studies [12].

Previous studies have identified differential phenotypes of aggregating and non-aggregating strains of *C. auris* [11, 13]. Whilst non-aggregating strains are more virulent, formation of large aggregates could be thought to complicate disinfection strategies. However, based upon the data presented in this and a previous study, this appears not to be the case, with minimal differences observed between the two phenotypes. Interestingly, our findings highlight potential species variation having regard to H₂O₂ and 0.05% CHX susceptibility. Both tested *C. auris* isolates were shown to be statistically more resilient to these treatments compared to *C. albicans* and *C. glabrata*. This is unlike previous disinfection studies [7, 12], whereby homogenous efficacy was shown across all tested *Candida* species, albeit tested in suspension, suggesting of a *C. auris* biofilm-related tolerance.

This study has its limitations in that we only assessed a small number of *Candida* isolates and that no physical application to disinfection (e.g. wiping) was included, to which could influence the survival of *C. auris*. Future studies assessing the efficacy of CHX diluted in alcohol may provide a potential anti-biofilm strategy for successful skin disinfection. Collectively these findings illustrate the need for a greater understanding of the survival strategies of *C. auris*. Given the documented high transmissibility between patients and the environment, the implementation of stringent infection prevention and control procedures, coupled with the biological understanding of the organism will ultimately aid the intervention strategies of this emerging pathogen.

Acknowledgements

We would like to thank Mrs Margaret Mullin (Integrated Microscopy Facility, University of Glasgow) for assistance with the SEM processing and imaging.

Ethical approval

Not required.

Funding

We would like to thank the European Society for Clinical Microbiology and Infectious Disease for financial support for LS.

Competing interests

None declared.

Author contributions

RK, EM, ET, LS and CD participated in study design, performed all experimental procedures and manuscript preparation. BJ and CW contributed to study design and preparation of the manuscript. GR conceived the study and was responsible for the approval of the final manuscript.

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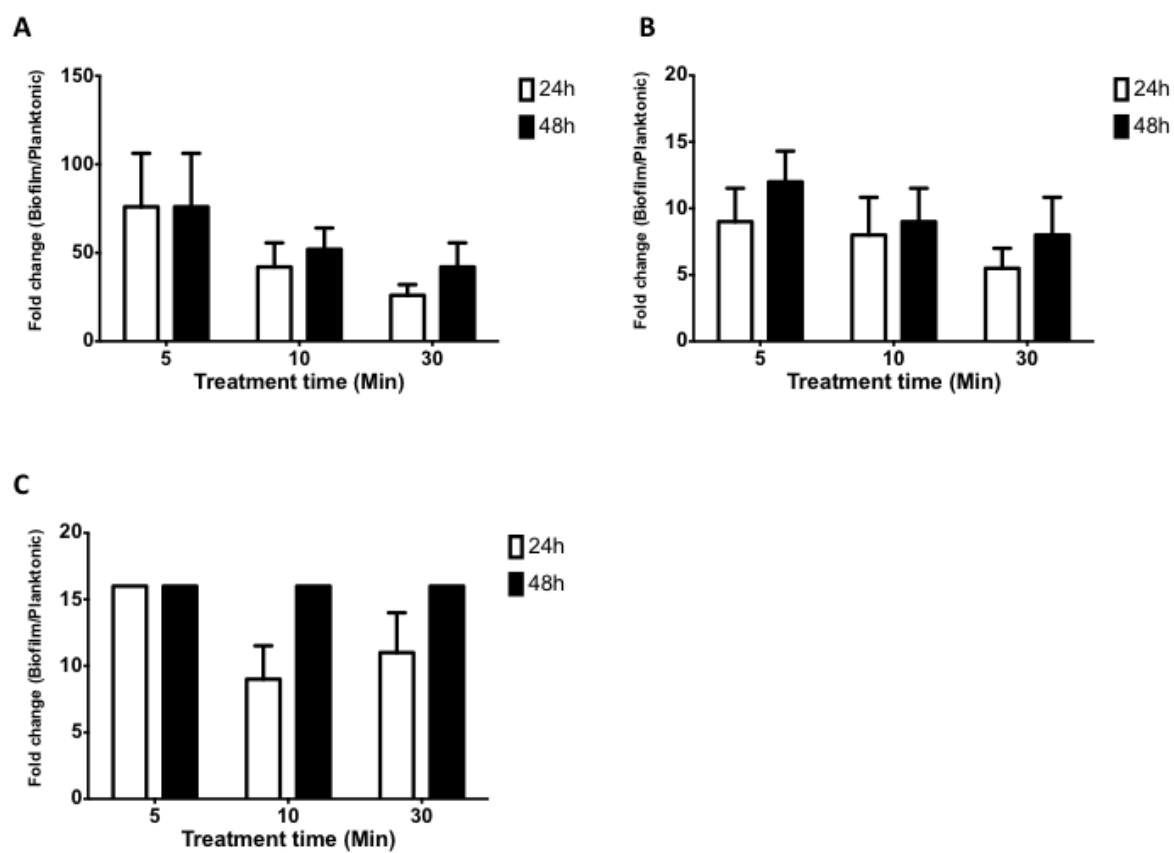
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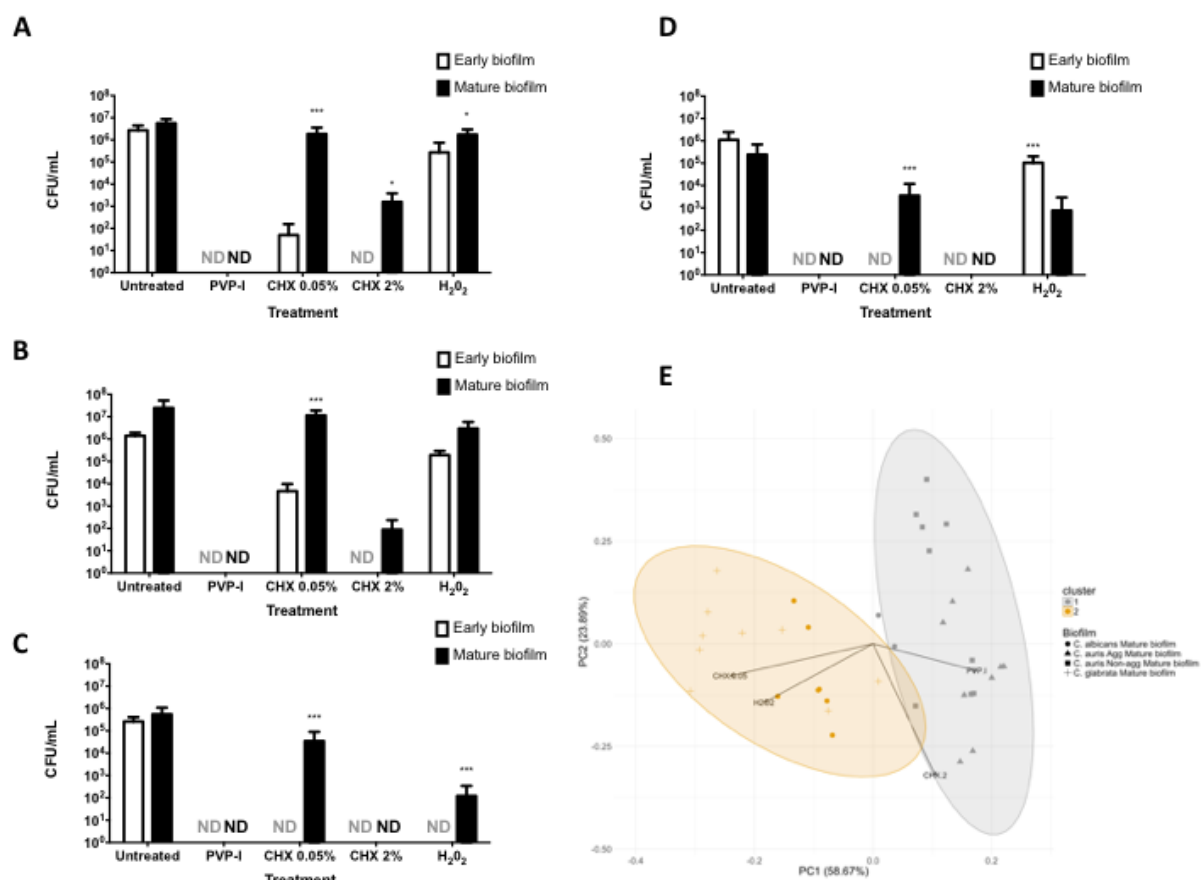
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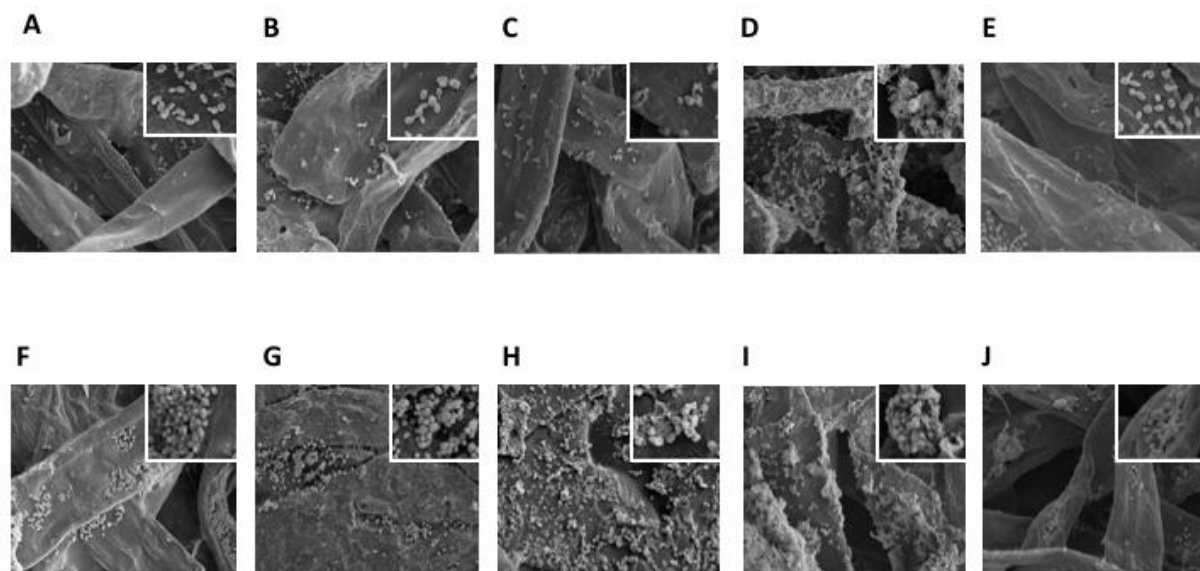
Figure 1. Fold-change comparisons between biofilm and planktonic MIC values of three antiseptics against *C. auris*. Contact time dependent sessile MICs for PVP-I (A), CHX (B) and H₂O₂ (C). MIC values are presented as the fold-change increase of biofilm compared to planktonic MICs, assessed using the XTT assay at a 90% reduction level in comparison to untreated control. Error bars represent the standard deviation of the means.

Figure 2. Biofilm sensitivities of *Candida spp.* to antiseptic treatment. Early (90 min) and mature (48 h) biofilms were treated for 5 min with each antiseptic. Viability analysis was performed using the Miles and Misra method for *C. auris* NCPF 8971 (A), *C. auris* NCPF 8978 (B), *C. albicans* ATCC 10231 (C) and *C. glabrata* ATCC 2001 (D). Principal component analysis of mature biofilms of each isolate with each treatment parameter (E). Data represents triplicate values from 3 independent experiments, with statistical analysis between log₁₀ reductions from the positive control of early and mature biofilms (* p<0.05, ***p<0.001, ND – not detectable).

Figure 3. Scanning electron micrographs of treated early and mature *C. auris* biofilms. *C. auris* NCPF 8971 biofilms were grown for 90 min (early) and 48 h (mature) before being fixed, processed and visualised using SEM. Early biofilms are shown in the upper panels A-E, with mature biofilms shown in the lower panels F-J. Treatments are indicated as follows: Untreated (A,F), 10% PVP-I (B,G), 0.05% CHX (C,H), 2% CHX (D,I) and 3% H₂O₂ (E,J). Images are view at ×1000 magnification, with ×5000 magnification (inset).







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